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Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms

(mobility shift of separated strands/point mutation/restriction fragment length polymorphism)

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ABSTRACT **We developed mobility shift analysis of single-stranded DNAs on neutral polyacrylamide gel electrophoresis to detect DNA polymorphisms. This method follows digestion of genomic DNA with restriction endonucleases, denaturation in alkaline solution, and electrophoresis on a neutral polyacrylamide gel. After transfer to a nylon membrane, the mobility shift due to a nucleotide substitution of a single-stranded DNA fragment could be detected by hybridization with a nick-translated DNA fragment or more clearly with RNA copies synthesized on each strand of the DNA fragment as probes. As the mobility shift caused by nucleotide substitutions might be due to a conformational change of single-stranded DNAs, we designate the features of single-stranded DNAs as single-strand conformation polymorphisms (SSCPs). Like restriction fragment length polymorphisms (RFLPs), SSCPs were found to be allelic variants of true Mendelian traits, and therefore they should be useful genetic markers. Moreover, SSCP analysis has the advantage over RFLP analysis that it can detect DNA polymorphisms and point mutations at a variety of positions in DNA fragments. Since DNA polymorphisms have been estimated to occur every few hundred nucleotides in the human genome, SSCPs may provide many genetic markers.**

The nucleotide sequences of DNAs in humans are not identical in different individuals. Nucleotide substitutions have been estimated to occur every few hundred base pairs in the human genome (1). Nucleotide sequence polymorphism has been detected as restriction fragment length polymorphism (RFLP). RFLP analysis of family members has been used to construct a genetic linkage map of the human genome (2, 3), and this analysis has also revealed the chromosomal locations of genetic elements involved in hereditary diseases such as Huntington disease (4), adult polycystic kidney disease (5), cystic fibrosis (6–8), Alzheimer disease (9, 10), and Duchenne muscular dystrophy (11, 12). Thus prenatal diagnosis of diseases such as cystic fibrosis is possible with RFLP probes. Recently, RFLP analysis has indicated specific loss of heterozygosity at particular loci on chromosomes in cancerous portions of tissues in several human cancers, including retinoblastoma, Wilms tumor, small cell carcinoma of the lung, renal cell carcinoma, bladder carcinoma, breast carcinoma, meningioma, acoustic neuroma (see ref. 13 for a review), colorectal carcinoma (14, 15), and multiple endocrine neoplasia type 1- or type 2-associated carcinomas (16, 17). This loss of heterozygosity suggests the involvement of recessive mutation of particular genes in development of these cancers.

Although RFLPs are very useful for distinguishing two alleles at chromosomal loci, they can be detected only when DNA polymorphisms are present in the recognition sequences for the corresponding restriction endonucleases or when deletion or insertion of a short sequence is present in the region detected by a particular probe. To identify DNA polymorphisms more efficiently, Noll and Collins used a simplified method of denaturing gradient gel electrophoresis (18) that had been developed by Myers *et al.* (19). As analysis of mobility shift [probably due to a conformational change of single-stranded DNAs on polyacrylamide gel electrophoresis (20)] has been used to detect point mutations (21), in this work we examined whether the mobility shift of single-stranded DNA caused by a single nucleotide substitution could be used to detect nucleotide sequence polymorphisms. The results indicated that mobility shift analysis is an efficient method for detecting DNA polymorphisms and for distinguishing the two alleles at chromosomal loci.

MATERIALS AND METHODS

Cell Lines. The human bladder carcinoma cell line T24 was obtained from the American Type Culture Collection. The human malignant melanoma cell line SK2 was established from a tissue that had been maintained in *nude* mice (22).

DNA Isolation. High molecular weight DNA was prepared from human leukocytes or cultured human tumor cell lines by the method of Blin and Stafford (23).

Plasmids. Plasmid pNCO106 was prepared by inserting a 2.9-kilobase pair (kb) *Sac* I fragment of the *HRAS1* gene from SK2 cells into pUC19 (24). Plasmid pT22 was constructed by inserting a 6.6-kb *Bam*HI fragment of the *HRAS1* gene from T24 cells into pBR322 (a gift from M. Wiglar, Cold Spring Harbor Laboratory).

Subcloning and Sequencing of DNA Fragments. From pNCO106 and pT22, a 371-base-pair (bp) *Pst* I fragment carrying exon 1 and a 298-bp *Pst* I fragment containing exon 2 of the *HRAS1* gene were isolated and subcloned into the pGEM-2 vector (Promega Biotec). The nucleotide sequences of the subcloned fragments were determined by the dideoxynucleotide method (25), using Sequenase (United States Biochemical) and the SP6 or T7 promoter primer (Promega Biotec).

Analysis of Single-Strand Conformation Polymorphisms (SSCPs). High molecular weight DNA (20 μg) was digested completely with restriction endonucleases under the conditions recommended by the suppliers. The reaction mixture was extracted once with phenol/chloroform (1:1, vol/vol) and once with chloroform. After addition of 0.1 vol of 3 M sodium acetate, DNA fragments were precipitated from the

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Abbreviations: RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

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aqueous phase by addition of 2.5 vol of ethanol. Strands were separated out by the method of Maxam and Gilbert (20) with a slight modification. DNA precipitates were dissolved in 20 μl of denaturing solution (0.3 M NaOH/1 mM EDTA) and then mixed with 3 μl of 50% (vol/vol) glycerol/0.25% xylene cyanol/0.25% bromophenol blue. The mixture was applied to a neutral 5% polyacrylamide gel (20 × 40 × 0.2 cm) with or without 10% glycerol in a well of 10 mm width and subjected to electrophoresis in 90 mM Tris-borate, pH 8.3/4 mM EDTA at 180 V for 12–36 hr at 17°C. DNA fragments in the gel were then transferred to a nylon membrane (Hybond-N, Amersham) by electrophoretic blotting in 0.025 M sodium phosphate, pH 6.5, at 1 A for 2 hr at 4°C by the procedure recommended by the membrane supplier. The membrane was then dried and baked at 80°C for 2 hr. Hybridization with ^{32}P-labeled DNA probes was performed in 50% (vol/vol) formamide/6× SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0)/10 mM EDTA/5× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.5% $NaDodSO_4$ containing denatured salmon sperm DNA at 100 μg/ml and 10% dextran sulfate at 42°C for 16 hr. The blots were washed twice in 2× SSC/0.1% $NaDodSO_4$ for 30 min at 65°C and then once in 0.1× SSC at 65°C for 10 min. Autoradiography was carried out at −80°C for 2–7 days by exposing the membranes to x-ray film (XAR-5, Kodak) with an intensifying screen (Cronex Lightning Plus, DuPont).

Analysis of RFLP. RFLP analysis was performed as described (26). High molecular weight DNA (5 μg) was digested with an appropriate restriction endonuclease and the digest was fractionated by electrophoresis in a 0.7% agarose gel.

DNA Probes for Hybridization. Cloned *Pst* I fragments 371 and 298 bp long carrying exon 1 and 2 of the normal human *HRAS1* gene (27), respectively, were used as specific probes for the corresponding exons. The 2.8-kb *Hin*dIII fragment isolated from phage 9D11 (28), provided by the Japanese Cancer Research Resources Bank, was used as a specific probe for the *D13S2* locus on human chromosome 13 (29). Probes were labeled to a specific activity of 2–10 × 10^8 cpm/μg by nick-translation (30) with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq) as a radioactive substrate.

RNA Probes for Hybridization. Single-stranded RNA probes were prepared by the method of Melton *et al.* (31) with plasmid constructs carrying the fragments used as DNA probes in the pGEM-2 vector as templates. RNA synthesis on each strand of the templates was carried out with T7 RNA polymerase (TOYOBO, Tokyo) or SP6 RNA polymerase (Amersham) in the presence of [α-^{32}P]UTP as a radioactive substrate. Concentration of UTP was adjusted to 500 μM by adding the nonradioactive nucleotide (final specific activity, 40 Ci/mmol) to ensure synthesis of full-length RNA copies. The hybridization conditions and washing procedures were the same as those for DNA probes.

RESULTS

Mobility Shift by Single Base Substitution. To determine whether a single base substitution altered the mobility of single-stranded DNAs on neutral polyacrylamide gel electrophoresis, we separated *Pst* I fragments carrying exon 1 or 2 of the human *HRAS1* gene, whose nucleotide sequences are known. In the human melanoma cell line SK2, one of the two alleles of the *HRAS1* gene is known to be activated by point mutation at codon 61 in exon 2 (32) and also amplified about 10-fold (33). The human bladder carcinoma cell line T24 has been reported to contain only one allele of the *HRAS1* gene, which carries a mutated codon 12 in exon 1 (34, 35). From plasmid constructs pNCO106 and pT22, containing the transforming allele of the *HRAS1* gene of SK2 and T24 cells, respectively, a 371-bp *Pst* I fragment carrying exon 1 of the gene was isolated and subcloned in the pGEM-2 vector. Similarly, a 298-bp *Pst* I fragment carrying exon 2 of the *HRAS1* gene was isolated from the same plasmid constructs and subcloned. By determination of the total nucleotide sequences of the subcloned fragments, we confirmed the single nucleotide substitution at codon 12 in the 371 nucleotides of the *Pst* I fragment between the SK2 gene and the T24 gene (GGC in the SK2 gene and GTC in the T24 gene). The nucleotide sequences of the 298-bp *Pst* I fragments carrying exon 2 of the SK2 and T24 genes were also confirmed to differ from each other by only one nucleotide in codon 61 (CTG in the SK2 gene and CAG in the T24 gene). After denaturation in alkaline solution, these cloned *Pst* I fragments were subjected to electrophoresis in neutral 5% polyacrylamide gel. The separated strands were then transferred to a nylon membrane by electrophoretic blotting and hybridized with ^{32}P-labeled DNA probes. As shown in Fig. 1*A*, the pair of separated strands of the *Pst* I fragment carrying exon 1 of the T24 gene (lane 2) moved slightly faster than those of the SK2 gene (lane 1). In the case of the *Pst* I fragment carrying exon 2, the mobilities of the separated strands of the SK2 gene (Fig. 1*A*, lane 3) were significantly different from those of the T24 gene (lane 4). Three bands were observed in the sample from the SK2 gene. Hybridization with single-stranded RNA probes showed that the bands with the fastest and the slowest mobilities were from the same strand of the fragment, while the middle band corresponded to the complementary strand (data not shown). Usually the slowest-moving band was the major one from the particular strand and the ratio of the slowest and the fastest bands varied depending on the conditions of electrophoresis, especially the temperature of the running gels. These results suggested that a particular single-stranded DNA could take at least two different molecular shapes, depending on the conditions of electrophoresis.

In the system containing homogeneous cloned DNA fragments, we could demonstrate mobility shift of single-stranded DNAs due to a single base substitution. To determine whether the same mobility shift could be observed in the presence of DNA fragments other than a target fragment, we digested genomic DNAs from the two tumor cell lines SK2 and T24 with *Pst* I and subjected the total digests to electrophoresis in neutral polyacrylamide gel after denaturation. As shown in Fig. 1*B*, the patterns of the separated strands of the fragments carrying exon 1 or 2 of the *HRAS1* gene from the genomic DNAs were essentially the same as those of the cloned fragments. This result indicated that the mobility shift due to a single base substitution of a single-stranded DNA fragment in total digests of genomic DNA

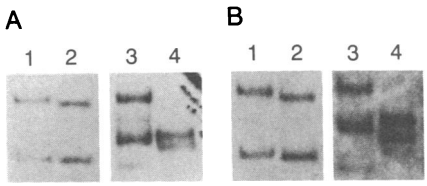

FIG. 1. Mobility shift of single-stranded DNA fragments due to a single base substitution. (*A*) Plasmid clones (2 pg) of fragments carrying exon 1 (371 bp) and exon 2 (298 bp) of the *HRAS1* gene from malignant melanoma SK2 cells (lanes 1 and 3, respectively) and from bladder carcinoma T24 cells (lanes 2 and 4, respectively) were digested with *Pst* I. (*B*) Total genomic DNAs (20 μg) from SK2 cells (lanes 1 and 3) and from T24 cells (lanes 2 and 4) were digested with *Pst* I. After denaturation, the fragments produced were subjected to electrophoresis in neutral polyacrylamide gel without glycerol. Single-stranded DNAs were transferred to a nylon membrane and hybridized with the ^{32}P-labeled DNA probe for exon 1 of the *HRAS1* gene (lanes 1 and 2 in *A* and *B*) and the probe for exon 2 of the gene (lanes 3 and 4 in *A* and *B*).

could be detected and was not influenced by the presence of a large amount of unrelated DNA fragments.

SSCP Analysis of Human DNA at the *D13S2* Locus. The above results encouraged us to apply the mobility shift of single-stranded DNA due to a single base substitution to detection of nucleotide sequence polymorphisms of a particular fragment and, as can be done with RFLPs, to distinguishing two alleles at chromosomal loci. As the mobility shift might be due to a conformational change of the single-stranded DNAs, we designated the polymorphisms detected by the method as SSCPs.

Leukocyte DNA samples from 19 individuals (10 unrelated and 9 in two families) were digested with *Hae* III, and SSCPs of the fragments obtained from a region of about 3 kb at the *D13S2* locus on chromosome 13 were analyzed. When the digests were subjected to electrophoresis without denaturation and hybridized with the ^{32}P-labeled 2.8-kb *Hin*dIII fragment as a specific probe for the *D13S2* locus, five distinct double-stranded DNA fragments (F1 to F5 in order of size) without any RFLP were observed in all DNA samples. The results on DNA samples 1 and 2 are shown in Fig. 2*A* as examples. In contrast with the double-stranded fragments, separated strands of the same DNA fragments showed SSCPs with considerable frequency. Representative results are shown in Fig. 2 *B–D*. When nick-translated DNA was used as a probe, SSCPs were apparently observed in at least one of the four fragments (F2 to F5) in all four DNA samples (Fig. 2*B*). The mobility shift of one of the strands of fragment F4 in sample 1 was especially marked. However, the mobility shifts of single strands in other fragments were small and therefore the difference of the shifts was not clear when both strands of the fragments were hybridized with the nick-translated probe. To overcome this disadvantage, RNA copies (RNA 1 and 2 in Fig. 2 *C* and *D*) of each strand of the *D13S2* DNA fragment were prepared separately and used as probes for hybridization. As shown in Fig. 2 *C* and *D*, with either the RNA 1 or RNA 2 probe SSCPs were clearly detected in all fragments except fragment F1. In Fig. 2*E*, the alleles distinguished by SSCPs are summarized. SSCPs found in fragment F2 by using the RNA 1 probe could distinguish alleles with three different mobilities, designated as "slow" (s), "fast" (f), and "very fast" (vf). In addition to these three alleles, the SSCP analysis of the other DNA sample shown in Fig. 3*A* revealed the presence of an allele with "very slow" (vs) mobility in the fragment. The SSCPs of the other fragments, F3, F4, and F5, could also distinguish at least two alleles with "slow" (s) or "fast" (f) mobility. Analysis of 19 DNA samples revealed that mobility shifts found in F4 and F5 were coincidental.

Mendelian Inheritance of SSCPs. To confirm that the observed SSCPs of the *Hae* III fragments of the region at the *D13S2* locus were due to allelic variants of true Mendelian traits, we analyzed the DNAs of nine individuals in two related families. In Fig. 3*A*, SSCPs of fragments F2, F3, and F4 and the alleles identified are indicated. In each family, the genotypes of the progenies were consistent with the parental genotypes.

Relationship Between SSCPs and RFLPs. The same 19 DNA samples analyzed for SSCPs were also subjected to RFLP analysis. The DNAs were digested with *Msp* I or *Taq* I and RFLPs were detected by hybridization with the ^{32}P-labeled DNA probe for the *D13S2* locus. Of the 19 DNA samples digested with *Msp* I, five samples (sample 2 in Fig. 2, data not shown, samples 2, 3, 5, and 8 in Fig. 3*B*) showed RFLP. By *Taq* I digestion, RFLP was observed in only one of the DNA samples (sample 2 in Fig. 2, data not shown). Therefore, RFLP analysis revealed heterozygosity at the *D13S2* locus in only 5 of 19 individuals, while with SSCP analysis heterozygosity at the locus was found in at least one of the four *Hae* III fragments in 18 of the 19 DNA samples. This fact demonstrates that SSCP analysis is a superior tool for detection of genetic polymorphisms.

Factors Affecting SSCP Analysis. The mobility shift of single-stranded DNAs with DNA polymorphisms observed on neutral polyacrylamide gel electrophoresis is most likely due to conformational variations of the molecules. The conformation of single-stranded nucleic acid is expected to be affected by environmental factors such as the temperature of the gel during electrophoresis, the concentration of electrophoresis buffer, and the presence of denaturing agents in gels. The mobility shift of the *Pst* I fragments carrying exon 1 of the *HRAS1* gene shown in Fig. 1*A* (lanes 1 and 2) was clearly observed on electrophoresis at 17°C but not prominently at 23°C (data not shown). The pattern of the separated strands

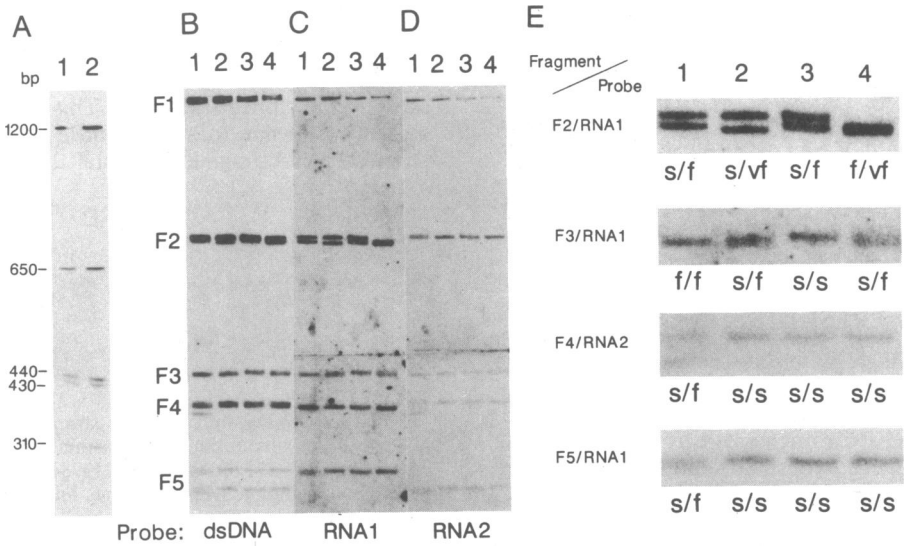

FIG. 2. SSCP analysis of human DNAs at the *D13S2* locus. DNA samples 1–4 were prepared from leukocytes of four unrelated individuals and digested with *Hae* III. The resultant fragments were subjected to electrophoresis in neutral polyacrylamide gel containing 10% glycerol before (*A*) and after (*B*, *C*, and *D*) denaturation. DNAs in the gel were transferred to a nylon membrane and then hybridized with the ^{32}P-labeled double-stranded DNA (dsDNA) probe for the *D13S2* locus (*A* and *B*) and with the ^{32}P-labeled single-stranded RNA probes for the *D13S2* locus (RNA1 in *C* and RNA2 in *D*). The five fragments produced from the *D13S2* region by *Hae* III digestion were designated as F1 to F5 in order of size. Alleles identifed by SSCPs are indicated in *E* with higher magnifications of informative fragments observed in *C* or *D*.

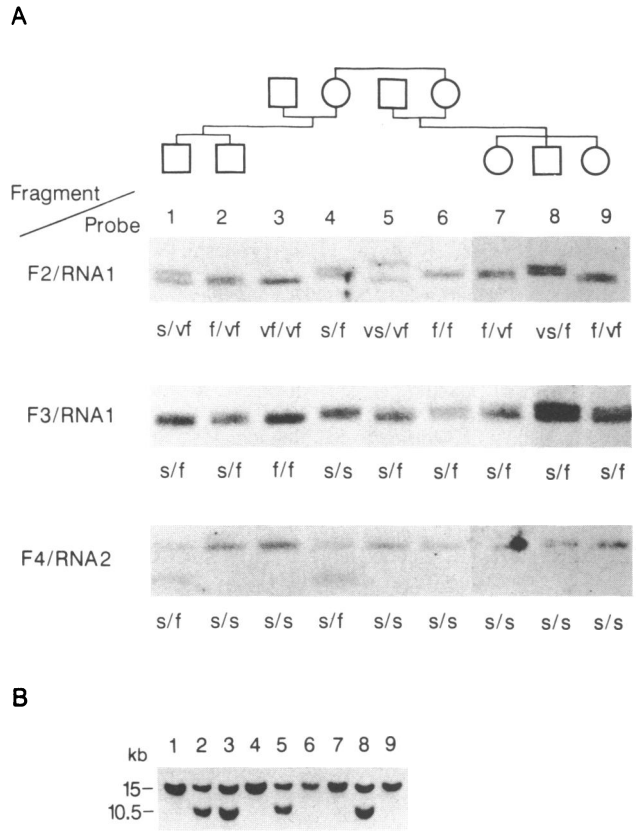

FIG. 3. SSCP and RFLP analyses of family members. (*A*) Leukocyte DNAs (20 μg) from the family members indicated at the top (○, females; □, males) were subjected to SSCP analysis using the *D13S2* probe as described in the legend for Fig. 2. As the mobility shifts found in fragments F4 and F5 were the same, the results with fragment F5 are not shown. (*B*) The leukocyte DNAs (5 μg) digested with *Msp* I were subjected to RFLP analysis using ^{32}P-labeled dsDNA as a probe for the *D13S2* locus.

of the fragments carrying exon 2 of the gene observed at 17°C and shown in Fig. 1*A* (lanes 3 and 4) was also altered at 23°C. Thus, the higher temperature might destroy some semistable conformations. The concentration of the running buffer also affected the mobility shift. When electrophoresis of the *Pst* I fragments analyzed in Fig. 1*A* was performed in a buffer of lower concentration (45 mM Tris–borate, pH 8.3/2 mM EDTA) at 17°C, the mobility shifts observed were similar to those at the higher temperature (23°C). Presence of 10% glycerol in gels also affected the mobility shift. However the effect of glycerol was rather complicated and mobility shifts due to DNA polymorphisms were often enhanced by this reagent. For example, the mobility shifts observed in Fig. 2 were enhanced when electrophoresis was performed in gel containing 10% glycerol. On the other hand, the mobility shift shown in Fig. 1 was reduced by the presence of 10% glycerol in the gel.

DISCUSSION

By neutral polyacrylamide gel electrophoresis, we could separate two single-stranded DNA fragments in which the nucleotide sequences differed at only one position. The mobility shift due to a single base substitution could be observed not only in cloned fragments but also in fragments of total genomic DNA after restriction endonuclease digestion. We applied the method to detect nucleotide sequence polymorphisms in human genomic DNA and could observe the mobility shift of single-stranded DNA by using a genomic sequence probe arbitrarily chosen. Single-stranded DNAs of the same nucleotide length can be separated by polyacrylamide gel electrophoresis, probably due to a difference in their predominant semistable conformations (20). The mobility shift of single-stranded DNAs with DNA polymorphisms observed on gel electrophoresis might also be due to conformational change, and so we designated the features of DNAs as SSCPs. We do not know whether nucleotide substitution at any position in a fragment can be detected by SSCP analysis, but DNA polymorphisms at a variety of positions in a fragment could cause a difference in its conformation and result in change in mobility of the single strands on gel electrophoresis. Therefore, we thought that DNA polymorphism could be detected more frequently by SSCP analysis than by RFLP analysis, and our experimental results revealed that this was in fact the case. Like RFLP analysis, SSCP analysis is simple and does not require complicated instruments or specialized techniques.

As we confirmed that the observed SSCPs were due to allelic variation of true Mendelian traits, SSCP analysis of DNA fragments could be a useful and simple method for elucidating the human genetic linkage map by studies on families. Because DNA polymorphisms have been estimated to occur once every few hundred nucleotides of the human genome (1) and SSCP analysis can reveal nucleotide substitutions at various positions in a fragment, any restriction endonuclease fragment with a nucleotide length suitable for strand separation may provide information for distinguishing two alleles. Therefore, in theory, on a nylon membrane carrying separated strands of all possible fragments of genomic DNA, DNA polymorphisms at any chromosomal locus can be detected by repeated hybridization of the membrane with a variety of probes.

SSCP analysis can also be used to locate genetic elements involved in hereditary diseases and to detect DNA aberrations in human cancers. Comparison of DNA fragments from cancerous portions of tissues with those from normal portions by SSCP analysis can reveal amplified alleles of particular genes and loss of heterozygosity at particular chromosomal loci. A remarkable advantage of SSCP analysis is that it can be used to detect point mutations at various positions in a fragment. Recently, by means of the DNA polymerase chain reaction (PCR), a DNA segment of a single cell or a single sperm has been amplified to an amount sufficient for analysis by hybridization (36). Our preliminary result suggested that SSCP analysis of DNA segments amplified by PCR technique could be useful for diagnosis of genetic aberrations.

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